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PRELIMINARY FRACTIONATION OF TIGER RATTLESNAKE (CROTALUS TIGRIS) VENOM

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Running Title: Fractionation of Tiger Rattlesnake Venom

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ABSTRACT

S.A. Weinstein and L.A. Smith Preliminary fractionation of tiger rattlesnake (*Crotalus tigris)* venom. Toxicon --, ----, 19--. Tiger rattlesnake (Crotalus tigris) venom was fractioned by using fast protein liquid chromatography (FPLC). The crude venom had low protease activity, lacked hemolytic activity and had an i.p. LD_{50} of 0.070 mg/kg for mice. Lethal fractions obtained by anion and cation exchange were examined for antigenic identity with crotoxin and mojave toxin. Four toxins were obtained by anion exchange chromatography which showed immunoidentity with these toxins, and one fraction caused rear limb paresis in mice. A lethal toxin (about 10% of total venom protein) purified further with Superose-12 FPLC (molecular sieve) had an i.p. LD_{50} of 0.050 mg/kg for mice, reacted strongly with anti-crotoxin and anti-mojave toxin antiserum in ELISA and immunoelectrophoresis. This toxin also showed complete immunoidentity with crotoxin and mojave toxin in immunodiffusion assays with anti-crotoxin antiserum. The results indicated the presence of crotoxin and/or mojave toxin isoforms in this venom. Although this species has a low venom yield (average 10 mg per snake), the venom is highly toxic and contains high concentrations of several neurotoxic isotoxins.

The tiger rattlesnake Crotalus tigris (KENNICOTT, 1859) is a montane crotaline species with a range restricted to south central Arizona and north central Sonora to Guaymas, Sonora, Mexico. It is a medium- sized rattlesnake species (65-75 cm average) with several morphological specializations, most notably disproportionate body girth compared to head size, slender fangs, large rattle, and strongly banded pattern. GITHENS and WOLFF (1939) considered C. tigris venom as neurotoxic with two distinct neurotoxin effects (early respiratory failure followed by paresis). KLAUBER (1956) placed C. tigris in a North American crotaline group which consisted of C. scutulatus, C. mitchelli mitchelli and Sistrurus catenatus, all of which secreted distinctively potent venoms when compared with other North American rattlesnakes. MINTON and WEINSTEIN (1984) reported C. tigris venom as the most toxic rattlesnake venom exceeded potentially only by some populations of C. d. terrificus and C. scutulatus. WEINSTEIN *et al.* (1985) reported the presence of a toxin antigenically related to mojave toxin in C. tigris venom. The present study describes the fractionation of C. tigris venom and the isolation of two lethal toxins.

Crotalus tigris venom was obtained from two adult specimens collected in the vicinity of Portal, Arizona. Both specimens (one male, one female) were extracted for venom every 3 weeks. Freshly extracted venom was immediately frozen and lyophilized. A single sample of C. tigris venom (analyzed separately) was kindly supplied by James L. Glenn (Veterans Hospital, Venom Research Laboratory, Salt Lake City, UT).

Mojave toxin was purified from C. scutulatus scutulatus venom by the method of WEINSTEIN *et al.* (1985) and antibody against the toxin raised in female New Zealand white rabbits (2.2 kg) according to the immunization protocol of WEINSTEIN *et al.* (1985). Antibody against crude C. scutulatus scutulatus venom (obtained from a single male specimen from Yuma, AZ) was prepared by the same protocol.

Monoclonal antibody against crotoxin(KAISER and MIDDLEBROOK,1988) was kindly supplied by Dr. John L. Middlebrook. Enzyme-linked immunosorbent assays (ELISA) were carried out essentially as described by HENDERSON and BIEBER (1986). Immunodiffusion and immunoelectrophoresis were performed as described previously (WEINSTEIN *et al.* 1985).

Protein content was determined using the bicinchoninic acid assay (BCA assay, Pierce Chemicals, Rockford, IL) (SMITH *et al.*, 1988). Venom protease activity was measured using a commercial casein substrate (Bio-Rad Laboratories, Richmond, CA). The casein was incorporated in agarose, wells punched in the agarose and an appropriate venom dilution (from 0.125 to 1 mg/ml in phosphate- buffered saline(PBS),(0.05 M, pH 7.2) was applied. A trypsin solution (232 units/mg, 5 mg per assay, Millipore Corp., Freehold, NJ) served as a positive control and reference while a well filled with PBS served as a

negative control. Hemolytic activity was assayed following the protocol of BERNHEIMER *et al.*, (1975).

The i.p. LD_{50} of the crude venom was obtained by injection of male Swiss-Webster mice (19-21 g) in six groups of four mice per group. All injections were administered in the lower quadrants of the abdomen. Each dose was derived from a 10 mg/ml solution appropriately diluted in PBS (0.05 M, pH 7.2). The i.p. LD50 of isolated toxin fractions was determined by injection of male Swiss-Webster mice (18-20 g) in four groups of two mice per group. Each dose was injected directly from concentrated fractions in Tris-HCl containing 0.7 M NaCl. Several animals were injected with buffer alone as controls. Animals were observed after injection and mortality recorded after 24 hr. Animals succumbing to venom injections were necropsied and any gross tissue pathology examined and noted. The LD_{50} was calculated by the Spearman-Karber method (WORLD HEALTH ORGANIZATION, 1981).

Venom samples were prepared for fractionation by dissolving 10.5 mg in 600 ul of 0.05 M Tris-HCl, pH 7.0. The sample was centrifuged at 100,000 x g in an Eppendorf 5415 benchtop centrifuge (Eppendorf Labs.) Initial fractionation of 550 ul of centrifuged venom was performed using an FPLC System (Pharmacia) with a Mono-Q 10/10 (anion exchange, 10 cm x 10 cm) column. The column was eluted with a 0 to 1 M NaCl gradient in 0.05 M Tris-HCl, pH 7.0. Fractions of 2 ml were collected with a flow rate of 3.0 ml/min. The column eluate was monitored by absorbance at 280 nm and corresponding peak fractions of interest were pooled for further study. Fractions corresponding to lethal activity were pooled and concentrated by Amicon Ultrafiltration using a Ym-5 membrane (molecular weight cut-off = 5,000 daltons). The concentrated lethal pools (average volume 1.5 ml) were filtered through a millipore membrane (0.2 µ) and applied in 550 µl aliquots to a Superose 12 H_r 10/30 (molecular sieve) FPLC column. The column was developed with Tris-HCl, 0.05 M containing 0.7 M NaCl. Each run was performed at a flow rate of 0.5 ml/min and 1 ml fractions were collected. The eluate was monitored at 280 nm and corresponding fractions of high absorbance were pooled and examined for lethal activity. Cation exchange was performed with a Mono-S 10/10 (10cm X 10cm) column. Crude venom was prepared and fractioned as described above.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed in a Protein II mini-gel apparatus (Bio-Rad Laboratories) using a 12.5% gel according to the protocol of LAEMMLI (1970). Gels were run at 100 V, 25 mA for 60-65 min, then were fixed and stained with either silver nitrate or Coomassie blue R-250.

Two adult C. tigris produced on average pooled sample of 20 mg. The protein

content of the crude venom was 96-98%. The murine i.p. LD_{50} of crude C. tigris venom was 0.070 mg/kg (Table I). Animals succumbing to injections of crude venom exhibited tachypnea followed by a brief hyperactive period in which both dexterogyrate and sinistrogyrate rotations around the animal's longitudinal axis were observed. Several animals exhibited piloerection and almost all showed flaccid paresis. Cardiopulmonary death was preceded by prostration accompanied by spasms and/or fasiculations. Gross necropsy indicated some pulmonary congestion and several animals showed slight, diffuse hemorrhage. The venom in concentrations up to 500 µg lacked direct hemolytic activity. Assay for protease activity with a commercial casein substrate detected low, but significant, proteolytic activity (Table I). The crude venom showed approximately 10 bands in SDS-PAGE profiles, when stained with silver nitrate (Fig. 1).

Application of 10.5 mg of centrifuged crude venom onto a Mono-Q (anion exchange) column resulted in approximately 15 peaks (Fig. 2). Peaks E, H, J and K (fractions 17, 19, 23 and 24, respectively) contained lethal activity. Animals injected with 5-7 µg of protein from peaks H or J, or 10 µg of peak K protein, exhibited rapid prostration with tachypnea followed by rapid cardiorespiratory death. Peak E (fraction 17) was found to produce symptomology similar to that observed with peaks H-K, except hind- limb paresis was observed in several animals. Necropsy of animals succumbing to any of these peak fractions was unremarkable. Cation (Mono-S) exchange of crude C. tigris venom resulted in a profile which contained nine peaks (Fig. 3). Animals injected with 10-12 ug of protein from peaks A, B, C, and H succumbed rapidly with symptomology resembling closely that observed with Mono-Q peaks H-K. Mono-S peak F caused prostration and respiratory distress, but was not fatal in 10 ug doses. Due to the low venom yield, only two major toxin species were purified further. Superose-12 (molecular sieve) analysis of Mono-Q peak H, resolved this peak into four species (data not shown). The main species in doses of 125 ug/kg caused rapid prostration, loss of righting reflex, flaccid paresis and death. Necropsy did not show any remarkable gross pathology.

Superose-12 analysis of Mono-Q peak K resulted in resolution of three species (Fig. 4). The main peak fraction (fraction 8) caused tachypnea, rapid prostration accompanied by "in-pouching" of inguinal region, followed by cardiorespiratory death. Pulmonary congestion and minor hemorrhage was noted on necropsy. The i.p LD_{50} of this peak, which represented about 10% of total venom protein, was found to be 0.050 mg/kg (Table 1).

SDS-PAGE analysis of Superose-12 fraction 8 indicated two major and one minor band. The major bands had reduced M_r of 16,000 and 14,000, respectively (Fig. 1). SDS-PAGE analysis of the material in Mono-Q peak E indicated two bands with reduced

molecular weights of 16 and 44 kD, respectively (Fig. 1).

Immunological survey of crude C. tigris venom indicated antigenic identity between C. tigris venom and polyclonal antibody prepared against purified mojave toxin. Immunoelectrophoresis showed identical anodal migration of precipitin bands resulting from reacting venoms of C. tigris and C. d. terrificus with anti-mojave toxin antiserum. Other shared arcs were observed when these venoms were reacted with antiserum against crude venom of Crotalus scutulatus scutulatus (Fig. 5). Mono-Q peaks E, H, J and K, and Mono-S peaks A, B, C and H, all reacted strongly in ELISA with polyclonal antibody against mojave toxin and monoclonal antibody against crotoxin. The Superose-12 major species resolved from Mono-Q peak K reacted strongly with both antibody preparations. Ouchterlony double diffusion indicated 100% identity between C. tigris crude venom, Mono-Q peak K and crotoxin and mojave toxin. The crude venom also produced precipitin bands when reacted with anti-crotamine polyclonal antibody. These bands indicated complete immunoidentity between the reacting antigen of the venom and authentic crotamine from venom of Crotalus durissus terrificus.

The lethality, apparent molecular weight and immunoidentity of the toxins isolated by anion exchange from C. tigris venom suggest the presence of crotoxin isoforms in this venom. FAURE and BON (1987) described the presence of up to 15 different crotoxin isoforms from venom of Crotalus durissus terrificus. The isotoxins were present in varying concentrations in different venom samples and indicated microheterogeneity in individual snakes. Other investigators have demonstrated crotoxin homologs from venom of Crotalus vegrandis (KAISER and AIRD, 1987) and mojave toxin isotoxins from individual specimens of Crotalus scutulatus scutulatus (JOHNSON and BIEBER, 1988).

The rotations and convulsive movements observed in animals injected with crude venom is suggestive of the presence of a gyroxin-like toxin in this venom. Gyroxin from Crotalus durissus terrificus venom is a heat labile toxin with an M_r of 33-35 kD (SEKI *et al.*, 1980). Da SILVA *et al.* (1989) have demonstrated a lethal gyroxin analog from venom of Lachesis muta muta. The venom studied in the present investigation contained higher protease activity then that reported previously (MINTON and WEINSTEIN, 1984). This may indicate population variation in venom components as is observed with other ophidian venoms. The high lethal index of C. tigris venom and concommitantly high proportion of lethal toxins in this venom classifies this species as one to approach with extreme caution, regardless of the low venom yield. This species is also irritable and quick to strike upon provocation.

We are currently sequencing the major lethal fractions of C. tigris and examining minor fractions of the venom in order to characterize other toxins present in this most lethal of

rattlesnake venoms.

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LEGENDS

Fig. 1. SDS-PAGE OF CRUDE VENOM AND VENOM FRACTIONS OF C. tigris. Polyacrylamide gels (12.5%) were cast and run using a BioRad mini Protein II unit according to the method of LAEMMLI (1970). Apparent molecular weights were determined by Andrews plots. Proteins were detected by staining with 0.1% Coomassie Brilliant Blue R-250 or silver nitrate. Lane 1 - Mono-Q (anion exchange) FPLC fraction pool (fraction 17) of peak E; lane 2 -Superose FPLC (molecular sieve) fraction pool of major/lethal species (Mono-Q peak K) from C. tigris venom; lane 3 - crude C. tigris venom; lane 4 - molecular weight markers: phosphorylase b = 94,000, bovine serum albumin = 67,000, ovalbumin = 43,000, carbonic anhydrase = 30,000, soybean trypsin inhibitor = 20,100, α-lactalbumin = 14,400.

Fig. 2. CHROMATOGRAPHIC PROFILE OF CRUDE VENOM OF C. tigris SUBJECTED TO FPLC UTILIZING ANION EXCHANGE.
Crude venom (10.5 mg) was dissolved in Tris HCl 0.05 M, pH 7.0 and centrifuged at 100,000 g for 3 min. The supernate was then subjected to anion exchange using a Mono-Q HR 10/10 column attached to a FPLC unit (Pharmacia). The column was equilibrated with the Tris buffer and sample (550 µl) was injected onto the column. Chromatography was carried out with a flow rate of 3 ml/min and elution was accomplished witha linear gradient established using 1 M NaCl in the Tris buffer. Fractions of 2 ml were collectged and monitored for absorbance at 280 nm and lethal concentrated by Amicon ultrafiltration, and examined in SDS-PAGE.

Fig. 3. CHROMATOGRAPHIC PROFILE OF CRUDE VENOM OF C. tigris SUBJECTED TO FPLC UTILIZING CATION EXCHANGE.
Crude venom (10.5 mg) was dissolved in Tris HCl 0.05 M, pH 7.0 and centrifuged at 100,000 g for 3 min. The supernate was then subjected to anion exchange using a Mono-S HR 10/10 column attached to a FPLC unit (Pharmacia). The column was equilibrated with the Tris buffer and sample (550 µl) was injected onto the column. Chromatography was carried out with a flow rate of 3 ml/min and elution was accomplished with a linear gradient established using 1 M NaCl in the Tris buffer. Fractions of 2 ml were collected and monitored for absorbance at 280 nm and lethal activity concentrated by Amicon ultrafiltration, and examined in SDS PAGE.

Fig. 4. FPLC MOLECULAR SIEVE CHROMATOGRAPHY OF FPLC ANION EXCHANGE (MONO-Q) LETHAL PEAK K FRACTION POOL.

Molecular sieve chromatography was performed by using a Superose 12 HR 10/30 column attached to the FPLC unit. Elution was carried out with Tris HCl 0.05 M, pH 7.2 containing 0.7 M NaCl. A flow rate of 0.5 ml/min was maintained and fractions of 1.0 ml were collected. All fractions were monitored for absorbance at 280 nm and lethal activity.

Fig. 5. IMMUNOELECTROPHORESIS OF C. tigris and C. durissus terrificus VENOMS WITH ANTI-MOJAVE TOXIN ANTISERUM AND ANTI C. s. scutulatus CRUDE VENOM ANTISERUM.

Immunoelectrophoresis was perfomed at room temperature on 1% agarose-coated slides in Tricine buffer (0.028M, pH 8.6). Venom samples (100 ug) were electrophoresed at 12-16 mA for 45-50 min, developed with 0.1 ml antiserum, washed repeatedly, dried and stained with amidoschwarz.

Pattern A - center trough, anti-Mojave toxin serum; upper well, C.d.terrificus venom; lower well, C.tigris venom.

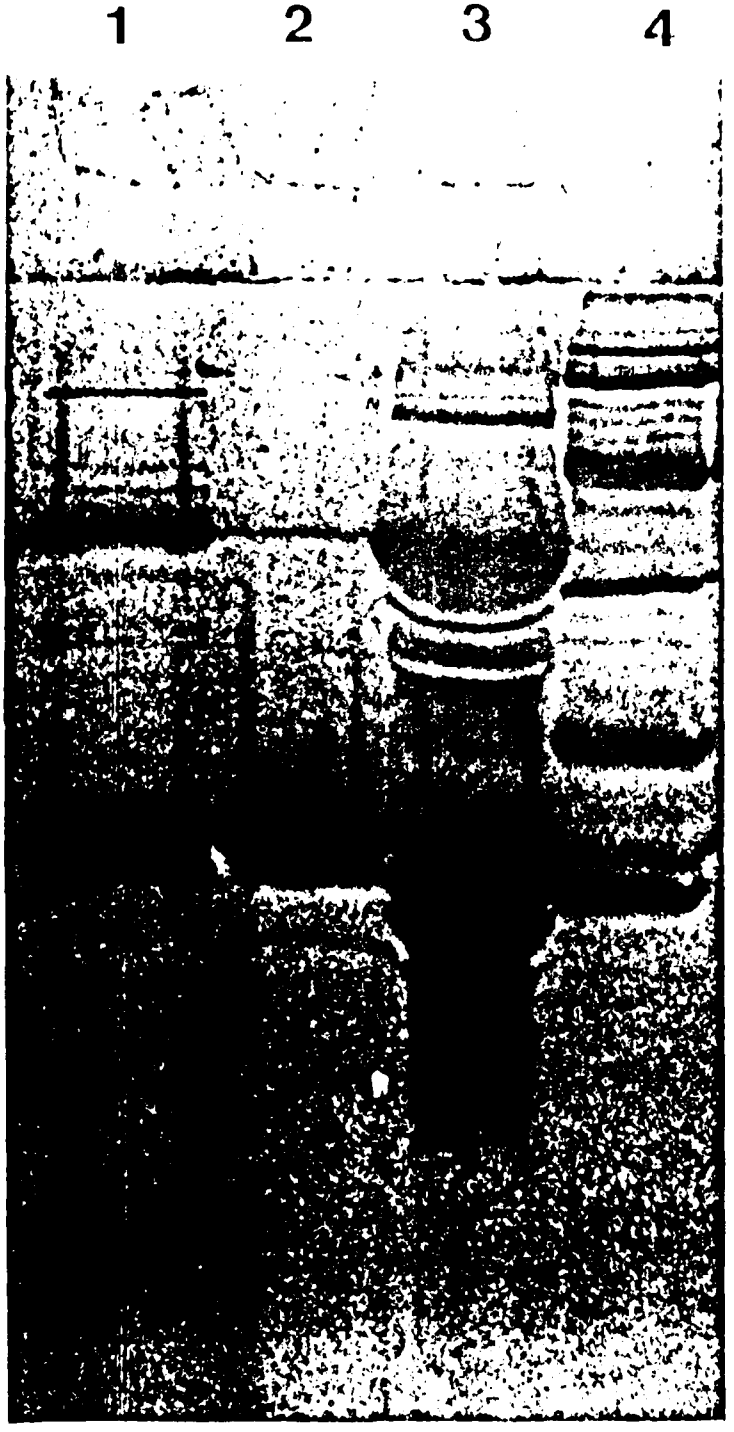
Pattern B - center well, Crotalus tigris venom; upper trough, anti-Mojave toxin serum; lower trough, anti-crude C. scutulatus venom serum.

Table I. Purification , Activity and Recovery of C. tigris Major Lethal Toxin

SAMPLE	PROTEIN	TOTAL VOLUME	TOTAL PROTEIN	MURINE i.p. LETHALITY (LD_{50})	[1] SPECIFIC ACTIVITY	% PROTEIN RECOVERY	[2] PROTEASE ACTIVITY
Crude Venom	10.5 mg / 0.5 ml	1.0 ml	21 mg	0.070 mg / kg	14.3	100 %	5 % <
Concentrated Superose-12 Pool	0.540 mg / ml	3.5 ml	1.9 mg	0.050 mg / kg	20	9 %	0

[1] Specfic activity = lethality / protein = 300 LD_{50} / 21 mg crude venom = 14.3

[2] Percentage clearing of casein substrate compared to 5 ug of trypsin standard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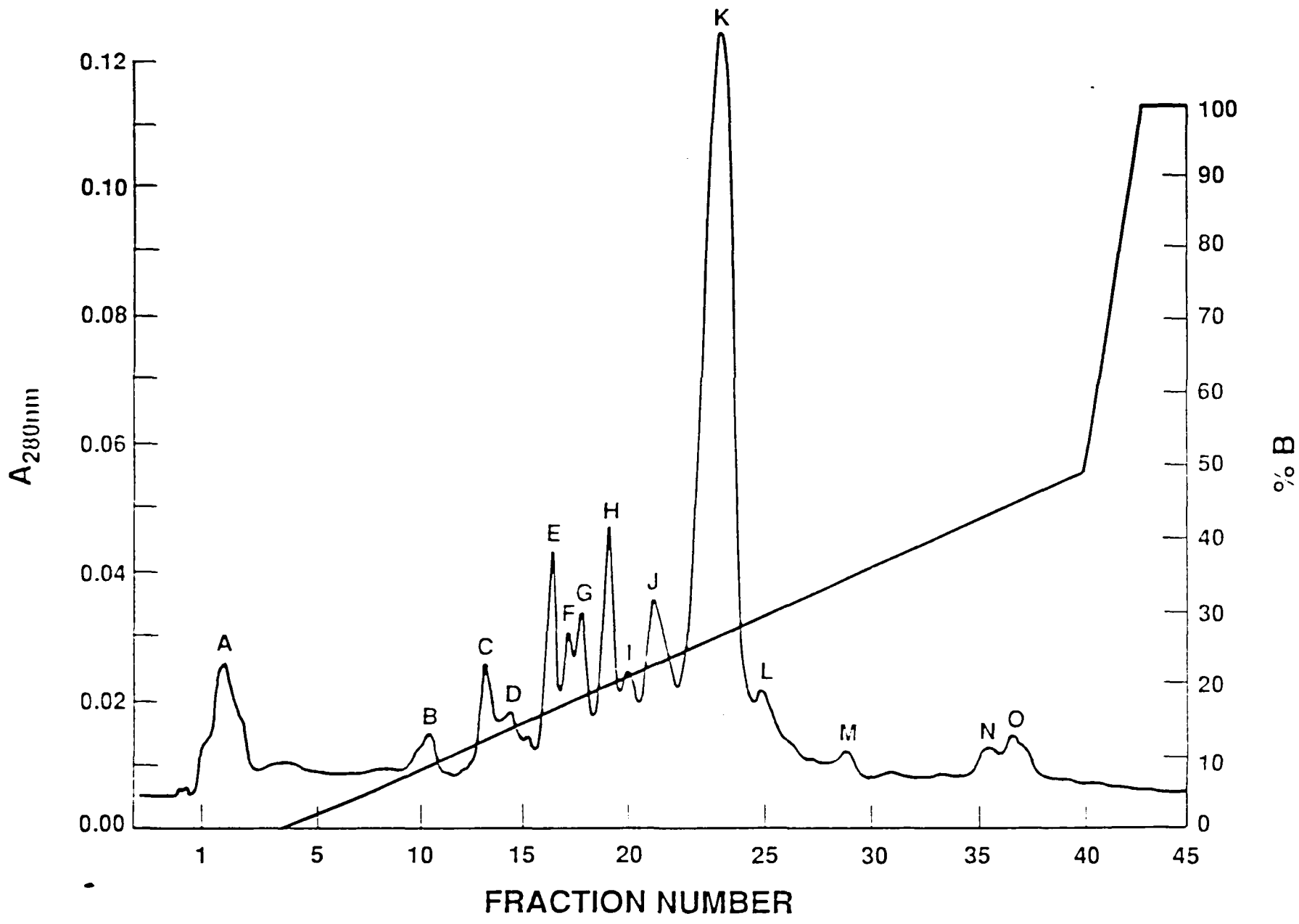
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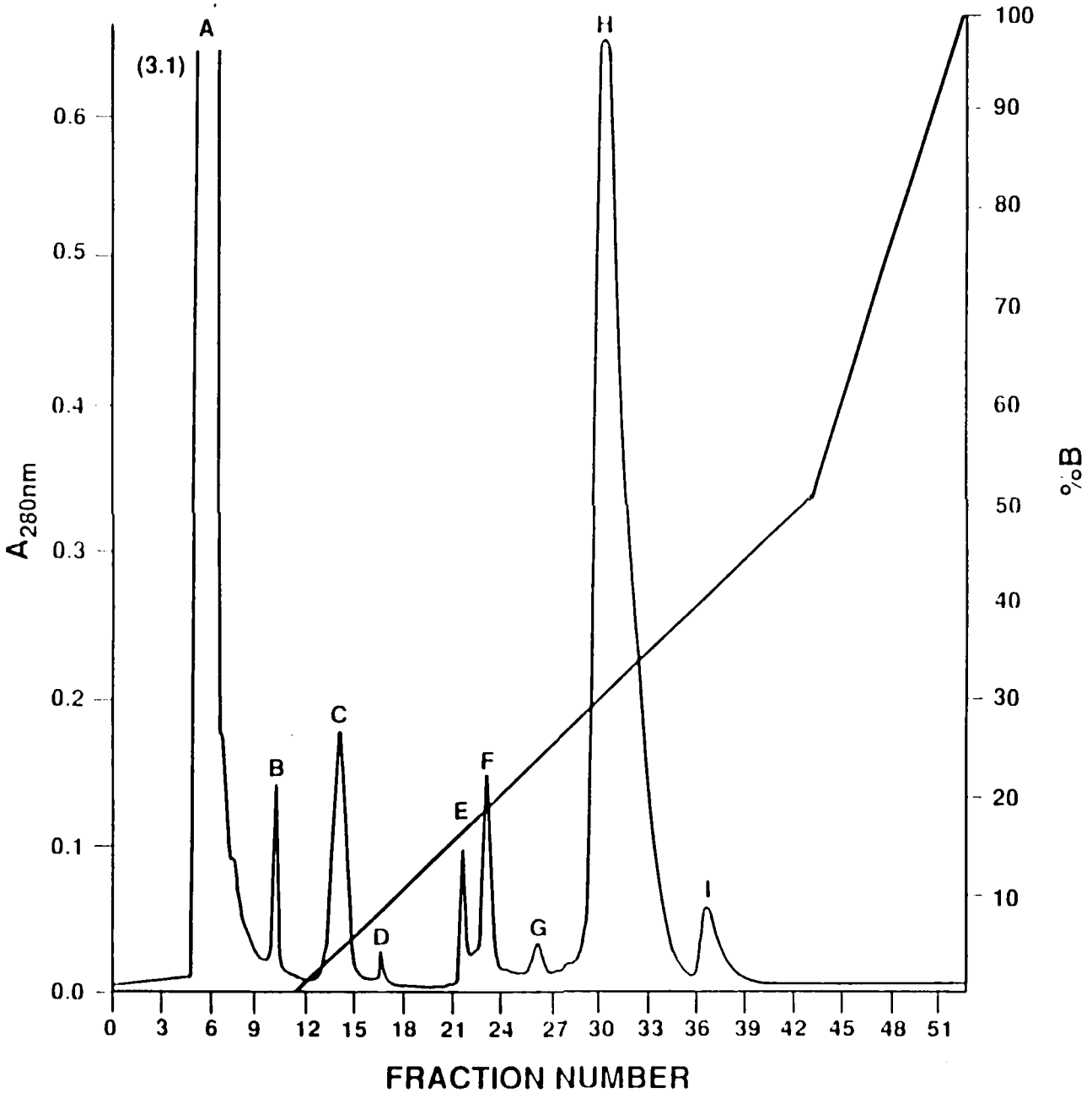
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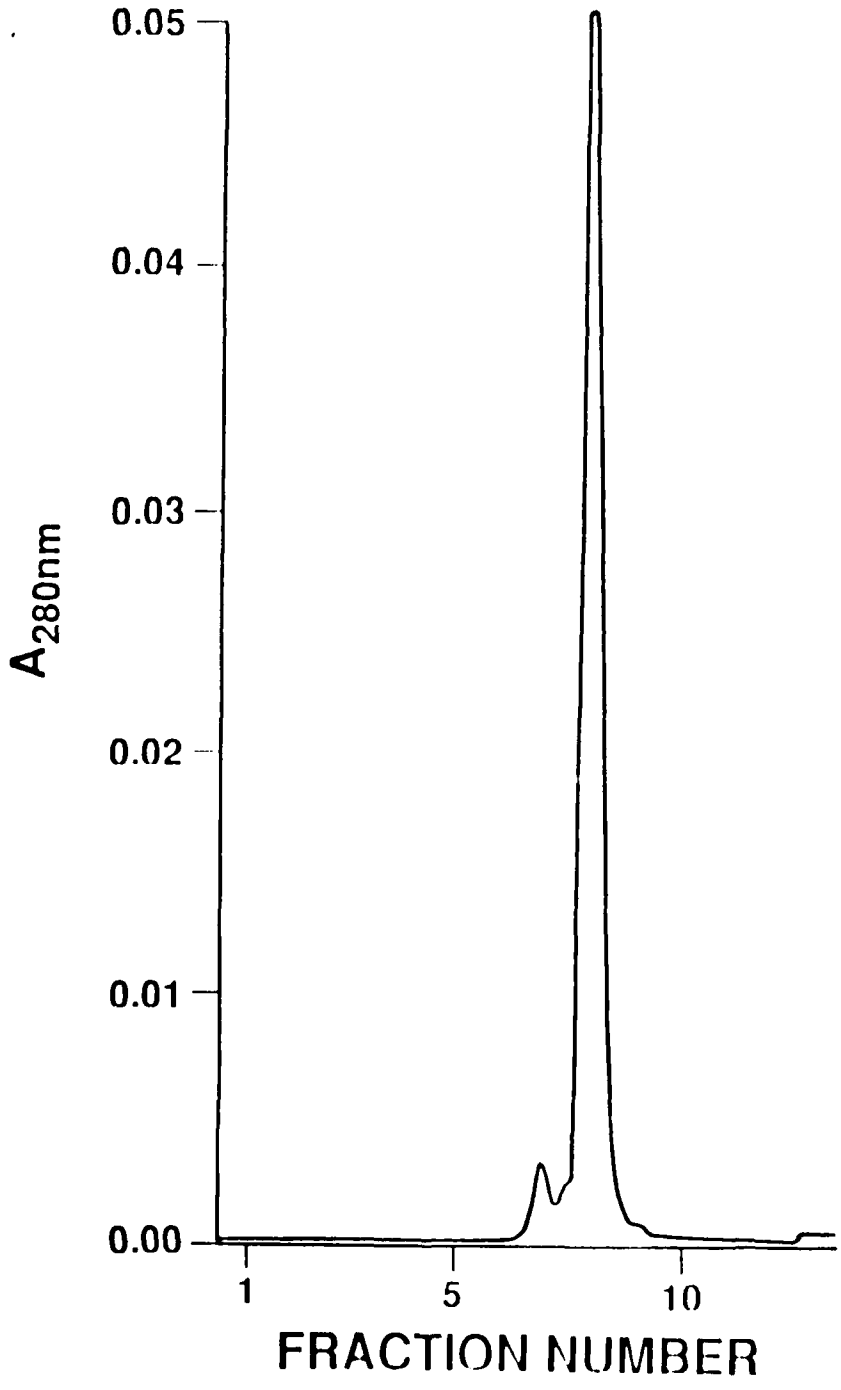
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